UNIVERSITI PUTRA MALAYSIA

PREVALENCE AND MOLECULAR CHARACTERISATION OF VANCOMYCIN RESISTANT ENTEROCOCCI (VRE) ISOLATED FROM BEEF

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By

NIMITA HASMUKH FIFADARA

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

August 2001

Dedicated to Taj and my parents

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman: Professor Dr. Gulam Rusul Rahmat Ali

Faculty: Food Science and Biotechnology

The present study was to isolate VRE from imported beef. In Malaysia, beef is the major consuming animal originated food and most of the beef is imported from those countries where the use of antibiotics in the feed of animals as a growth promoter was a common practice and was licensed. Out of 150 samples, 17 (11.3%) were positive for VRE. Sixty-seven (67) VRE were isolated from frozen imported beef (48) and burgers (19). The species identified were *E. faecium* (35), *E. faecalis* (22), *E. faecalis* asaccharolytic variant (3), *E. pseudoavium* (3), *E. gallinarum* (2), *E. maldoratus* (1) and *E. avium* (1). Various plating media and broths were evaluated for the isolation of VRE. Azide Dextrose broth (ADB) with vancomycin concentration of 50 µg/ml for 48 h enrichment and plating on Slanetz and Bartley agar (SBA) with vancomycin concentration of 50 µg/ml was concluded best for isolation of VRE. In the present study antibiotic resistance patterns and the rates of resistance of 67 isolates were evaluated. It

was observed that all the isolates were multiple resistant and resistant to ten of the sixteen antibiotics tested. All isolates were 100% resistant to streptomycin, vancomycin and teicoplanin. Other isolates were resistant between 94% to 97% to other eight antibiotics. Penicillin, ampicillin and chloremphenicol showed the least resistance namely, 26.8, 38.8 and 58.2%, respectively. Hemolytic activity on horse blood agar showed that 29 out of 67 isolates (43.3%) were β-hemolytic indicating to have potency to be pathogenic. The plasmid profiling revealed that 39 (58.2%) out of 67 bear plasmids of the range 1.0 to 35.8 MDa. Using specific PCR, *van*A gene was detected among 65 of 67 isolates (97%) which is considered to make these isolates resistant to vancomycin. The molecular epidemiology of *E. faecium* and *E. faecalis* using RAPD-PCR technique showed the difference in the genetic relatedness of the strains isolated from frozen imported beef and beef burgers. It showed the genetic relatedness in terms of % similarity from the dendrogram prepared between all the strains taken into study. RAPD-PCR gave high discriminating results between all the strains. The work clearly reveals that beef can be a vehicle for VRE in Malaysia. The need for intervention to control or eliminate antibiotic resistant *Enterococcus* from foods of animal origin has been made clearer by the results presented in this study.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi keperluan untuk Ijazah Doktor Falsafah

PREVALEN DAN PENCIRIAN MOLIKULAR ENTEROKOKI RINTANG VANKOMISIN (VRE) DARI PEMENCILAN DAGING LEMBU MENTAH DAN YANG DIPROSES

Oleh

NIMITA HASMUKH FIFADARA

Ogos 2001

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Dalam kajian yang dijalankan, adalah bertujnan untuk memencilkan VRE daripada daging lembu. Daging lembu dipilih sebagai sumber sampel kerena ianya dimakan oleh sebahagian besar populasi dan kebanyakan daging lembu diimpot dari negara-negara dimana antibiotik-antibiotik banyak digunakan dalam makanan haiwan untuk menggalakhan tumbesaran yang cepat. Enam puluh tujuh (67) VRE dipencilkan dari daging lembu mentah (87) dan yang telah diproses (63). Spesis-spesis yang dikenalpasti melalui ujian biokimikal secara konvensional adalah *E. faecium* (35), *E. faecalis* (22), *E. faecalis* asaccharolytic variant (3), *E. pseudoavium* (3), *E. gallinarum* (2), *E. maldoratus* (1) dan *E. avium* (1). Berbagai jenis media, brot dan masa pengayaan juga telah dioptimiskan untuk mencilan VRE. Azide Dextrose brot (ADB) dengan vankomosin pada

kepekatan 50 µg/ml selama 48 jam pengkayaan dan diplatkan atas Slanetz dan Bartley agar (SBA) dengan vankomosin pada kepekatan 50 µg/ml didapati sangat baik untuk pemencilan VRE. Corak kerintangan antibiotik menunjukkan bahawa pencilan-pencilan yang diperolehi sangat rintang terhadap kebanyakan antibiotik yang diuji dan aktiviti hemolitik diatas agar darah kuda memenjikan 29 daripada 67 pencilan (43.3%) adalah β-hemolitik menunjukkan mereka mempunyai potensi untuk menjadi patogenik. Profil plasmid menunjukkan bahawa 39 (58.2%) dari 67 membawa plasmid bersaiz antara 1.0 kepada 35.8 MDa. Dengan menggunakan kaedah PCR yang spesifik, gen *van*A dikesan didalam 65 dari 67 pencilan (97%), yang menyebabkan mereka rintang kepada vankomisin. Epidemiologi molikular *E. faecium* dan *E. faecalis* dengan menggunakan taknik RAPD-PCR unjukkan perbezann dari segi pertalian genetik pencilan-pencilan dari daging lembu mentah dan yang diproses. Ianya menunjukkan pertalian genetik dari segi % kesamaan dari dendrogram yang disediakan untuk untuh semua pencilan. RAPD-PCR memberi keputusan diskriminasi yang tinggi dikalangan semua pencilan. Adaleh dirumuskan bahawa daging lembu boleh menjadi satu pembawa VRE di Malaysia. Keperluan untuk pemantauan dan pengawalan kerintangan kepada antibiotik enterococcus yang berasal darihaiwan depat dilihat dengan jelas daripada keputusan yang rolehi dalam kajian ini.

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I certify that an Examination Committee met on 20th August 2001 to conduct the final examination of Nimita Hasmukh Fifadara on her Doctor of Philosophy thesis entitled "Prevalence and Molecular Characterization of Vancomycin Resistant Enterococci (VRE) Isolated from Beef" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relavant degree. Members of the Examination Committee are as follows:

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I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or any other institutions.

NIMITA FIFADARA

Date: 29 Aug' 2001

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviated	Full form
A	Adenosine
ABA	ABA (Asculin Bile Azide agar
ADB	Azide Dextrose Broth
Ap	Ampicillin
B	Bacitracin
BEAA	Bile Esculin Azide Agar
C	Cytosine
Car	Carbenicillin
CATC	Citrate Azide Tween Carbonate agar
Caz	Ceftazimide
CDC	Center for Disease Control
CHEF	Contour-clamped Homogenous gel electrophoresis
Cm	Chloremphenicol
D-Ala-D-Ala	D-alanyl-D-alanine
D-Ala-D-Ser	D-alanyl-D-serine
dATP	deoxy' Adenosine Triphosphate
dCTP	deoxy' Cytosine Triphosphate
dGTP	deoxy' Guanosine Triphosphate
DNA	DeoxyriboNucleic Acid
dTTP	deoxy' Thymine Triphosphate
EBVA	Enterococcosel broth with vancomycin
EDTA	Ethylenediamine Tetraacetate
Er	Erythromycin
fGTC	fluorogenic Gentamicin-Thallous Carbonate agar
G	Guanisine
g	gravity
Gm	Gentamycin
HLR	High Level Resistance
ID 32 Strep	Identificatin 32 Streptococcus Kit
*IS*1251	Insertion Sequence 1251
*IS*6770	Insertion Sequence 6770
KA	Crystal violet Azide agar
KAA	Kanamycin Asculin Azide agar
Kb	Kilo base
KDa	Kilo Dalton
KF	Kenner *faecalis* agar
Km	Kanamycin
Na	Nalidixic acid

Nor	Norfloxacin
LAB	Lactic Acid Bacteria
LAP	Leucine aminopeptidase
MAR	Multiple antibiotic resistant
MDR	Multi Drug Resistant
ME	M-enterococcus agar
µl	microlitre
µmol	micromole
mmol	millimole
MRS	de Man Rogosa and Sharpe
MRSA	Methicillin Resistant *Staphylococcus aureus*
P	PenicillinG
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
PyMS	Pyrolysis Mass Spectrometry
PYR	Pyrolidonylarylamidase
R	Resistant
RAPD	Random Amplification of Polymorphic DNA
RNA	RiboNucleic Acid
rpm	rotations per minute
S	Susceptible
SBA	Slanetz and Bartley Agar
ScS	Streptococci Selective agar
SF68	*Streptococcus faecium* 68
SFA	*Streptococcus faecalis* Agar
Sm	Streptomycin
T	Thymine
Ta	Annealing Temperature
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
Te	Tetracycline
Tec	Teicoplanin
Th	Sulfamethizole
TITG	Thallous Acetate Tetrazolium Glucose agar
Tn1546	Transposon 1546
Tn3	Transposon 3
TNF	Tumor Necrosis Factor
TSB	Tryptic Soya Broth
TSN	The Surveillance Network
V	Vancomycin
V.P.	Voges Prosker's test
vanA	gene A of vancomycin resistance (italic)
VanA	Phenotypic resistance to vancomycin (no italic)
VRE	Vancomycin Resistant Enterococci
WHO	World Health Organization

CHAPTER 1

GENERAL INTRODUCTION

Enterococci, especially *E. faecium* and *E. faecalis*, may be considered as opportunistic pathogens. Infections usually occur nosocomially in persons who are debilitated, have an underlying disease, or have received medical instrumentation. However the incidence of infections caused by enterococci, their seriousness, and the increasing difficulty of treating such infections because of multiple antibiotic resistance, put these organisms among the most important emerging human pathogens. Sources of enterococci involved in human infection were thought to be from the patient's endogenous microflora; however, person-to-person transmission of enterococci in hospital outbreaks as well as stool carriage of strains has been reported (Moellering, 1991; Jordens et al., 1994; Gordts et al., 1995; Gin et al., 1996).

As regular inhabitants of the intestine, enterococci may serve as indicators of fecal contamination, and are therefore of particular importance in food and public health microbiology. *E. faecalis* and *E. faecium* have been suspected, but remained unconfirmed, as causative agents of foodborne illness (Dack, 1956; Stiles, 1989). Several strains are used as probiotics and others are involved in a number of food fermentation for the production of certain cheeses and other fermented milk products. They are associated with natural fermentations such as in olives and fermented African products (Olasupo et al., 1994; Franz et al., 1997) and enterococci may become the predominant

population of in-package, heat-treated meats (Houben, 1982; Bell and DeLacey, 1984; Andre, Gordon and Ahmad, 1991). *E. faecalis* has assumed major importance in clinical microbiology as one of the leading causes of nosocomial infections, and both *E. faecium* and *E. faecalis* strains have developed resistance to most clinically used antibiotics, including the glycopeptide antibiotics vancomycin and teicoplanin. It is therefore important for food microbiologists to assess the significance of these bacteria in the foods.

Here the study is focused on whether pathogenic enterococci can be transmitted by foods and cause disease in a hospital setting, particularly with emphasis on vancomycin resistant enterococci (VRE). It was also thought that VRE originated in hospital environment and that they are disseminated to the community, but several researchers have proposed the opposite (Bates et al., 1993, 1994; Klare et al., 1995a,b; Das et al., 1997). A proposed source of VRE is farm animals in which there has been ergotropic use of avoparcin, a glycopeptide feed additive (Klare et al., 1995a,b; Das et al., 1997; Simonsen et al., 1998; Kruse et al., 1999). VRE have been isolated from a wide variety of farm animals, and these constitute an important reservoir of VRE that could be transmitted to hospital environment via contaminated meat (Klare et al., 1995a,b; Devriese et al., 1996). Chadwick et al. (1996) isolated VRE from chicken, pork and beef samples from retail markets in the UK and suggested that *van*A resistance genes may be introduced into the community via the food chain. VRE were also isolated from sewage, farm animals and uncooked chicken by Bates et al. (1994), and more importantly, they showed that blood and urine isolates from different hospital patients and a porcine isolate

shared the same ribotyping pattern. These findings strongly suggest that food transmission occurred and, as a result, two European countries (Denmark and Germany) banned the use of avoparcin (Morrison et al., 1997), followed by a European Union-wide ban (McDonald et al., 1997).

In the USA the situation with respect to nosocomial VRE infections appear to differ considerably from that in Europe, because avoparcin has not been licensed for use as a feed additive (McDonald et al., 1997). A community prevalence survey failed to isolate VRE from healthy volunteers without hospital exposures and from environmental sources or probiotic preparations (Coque et al., 1996). In contrast to Europe, transmission of VRE in the USA does not appear to be from the community to the hospital, and food has not been implicated as a possible vehicle for transmission. This raises the question of the source of VRE isolates in the USA. McDonald et al. (1997) proposed that undetected community transmission of VRE might occur at low levels. Alternatively, it was proposed that enterococci acquired vancomycin resistance genes from an unknown gastrointestinal bacterium (Rice, 1996; Morrison et al., 1997).

The important question is whether enterococci originating from food and community sources possess an equally pathogenic potential, or whether a difference in pathogenicity exists among enterococci from different sources. Using molecular characterization of resistance determinants for enterococci isolated from processed meat products and cheeses, Teuber et al. (1996) showed them either to be similar or identical to corresponding determinants known from clinical samples. Valdivia et al. (1996) showed

that the incidence of antibiotic resistance, as well as aggregation response to sex pheromones, was much higher in clinical strains than isolates from municipal wastewater. Regarding the food chain, it is not clear whether and with which frequency VRE strains are transferred.

It has been reported that strains of enterococci from dairy products do not produce hemolysin (Arihara et al., 1993; Giraffa, 1995), and it was suggested that absence of hemolytic activity should be a selection criterion for starter strains for dairy use (Giraffa, 1995). It is now known that hemolytic activity is not necessarily associated with all clinical isolates; therefore, absence of hemolytic activity in enterococci isolated from food does not mean that these bacteria are non-virulent. So, in this study we have carried out on the presence of VRE in frozen imported beef, local beef, frankfurters and burgers and analyzed the distribution of different VRE species. We also investigated, whether enterococci isolated from foods has any potential for virulence by examining the hemolysin production.

Antibiotic resistant enterococci have been isolated from foods such as raw milk cheeses, raw meats and sausages (Batish and Ranganathan, 1986; Knudtson and Hardtman, 1993b). Perreten and Teuber et al. (1995) showed that enterococci isolated from Salami and Landjager-types of fermented sausage were frequently resistant to streptomycin and lincomycin, while isolates from Emmental and Appenzeller cheeses showed a high frequency of resistance to erythromycin, gentamicin, tetracycline and/or